Iron delivery to cells is mediated by the interaction of plasma transferrin with cell surface transferrin receptors. Virtually all cells have transferrin receptors on their surface although, in the normal adult, about 80% of them are in the erythroid marrow. Circulating soluble transferrin receptors (sTfR) found in human plasma provide an estimate of the erythroid compartment mass. The concentration of sTfR is significantly increased in the presence of iron deficient anemia or enhanced erythropoiesis, but some controversy remains about its significance as a marker of iron status in overload conditions. Hereditary hemochromatosis (HH), a common genetic disorder of iron overload, constitutes a good model to address this question. HH is characterized by inappropriate increased iron absorption with progressive accumulation of iron in tissues and eventual organ damage. The large majority of HH patients are homozygous for a mutation in the HFE gene that causes a change in the HFE protein, from cysteine to tyrosine at position 282 (C282Y), impairing the normal association of HFE with $\beta_2$-microglobulin, therefore reducing its cell surface expression. Phenotypically, HH is recognized by abnormally high levels of transferrin saturation (TfSat) and serum ferritin, reflecting, respectively, increased iron absorption and iron accumulation in tissues. Subjects with the hemochromatosis phenotype, homozygous for the C282Y HFE mutation, exhibit a higher erythrocyte mass, as measured by the hemoglobin concentration, suggesting an increased iron uptake and hemoglobin synthesis by erythroid cells. Despite the strong association of the mutated HFE protein with iron overload, its role in the regulation of iron bal-
ance is not understood. The lack of hepcidin up-regulation in HH, supported by results in animal models, suggests that HFE may play an important part in the regulation of hepcidin expression. However, the functional relationship between HFE and hepcidin is still obscure. So far, the only confirmed link between the HFE–β2 microglobulin complex and other proteins of iron homeostasis is the observation that HFE binds with high affinity to the transferrin receptor.

Previous studies addressing the question of sTfR in HH patients have provided conflicting results. Independent studies by Huebers et al.14 and Baynes et al.,15 found no differences in sTfR between HH patients and controls. In contrast, Ledue and Craig reported that the serum concentration of sTfR is 50% lower in HH patients.16 One explanation for the discrepancies found could be the fact that the different studies include highly heterogeneous groups of patients, clinically characterized before the discovery of the HFE gene. Another problem could be the fact that not all patients were analyzed at the same stage of phlebotomy treatment. A direct and significant relationship between the number of phlebotomies and sTfR concentration was described in HH.17 Accordingly, an inverse correlation between the sTfR and serum ferritin or TfSat was observed in African iron overload.18 The objective of the present study was to characterize the relationship between the serum concentration of sTfR and other hematologic and biochemical parameters of iron homeostasis in HH patients homozygous for the C282Y mutation of HFE, and compare these with appropriate reference values for sTfR in relation to iron parameters in a normal healthy population.

Design and Methods

Subjects and study design

HH patients

Forty-five HH patients (29 males and 16 females; age range=24-77 years) were included in this study. All patients were diagnosed and are regularly followed-up at the Haemochromatosis Outpatient Clinic of Santo António General Hospital, Porto, where all the clinical information is recorded. Only patients genetically characterized as homozygous for the C282Y mutation of HFE were included. Clinical and genetic data from these patients have been published previously.14,15 For the purpose of the present study, measurement of sTfR was included as a routine procedure in the regular analytical evaluation of patients. During the study period, patients were at different stages of their treatment course. Fifteen patients were under intensive phlebotomy treatment: 10 of them at the beginning and 3 at the end of treatment, i.e., near iron depletion. The remaining 30 were receiving maintenance therapy. The inclusion of patients at different stages of iron load was important in order to allow an analysis of sTfR values in relation to a wide range of TfSat values. In addition, for the purpose of defining some individual serial profiles, we retrospectively analyzed the sTfR in frozen stored samples collected at regular intervals during the treatment period in three patients: two patients (one male and one female aged, respectively, 45 and 60 years) who were followed-up from diagnosis until one year after the end of intensive treatment, and one patient who presented with anemia and was followed-up regularly during the first six months of intensive phlebotomy treatment. All clinical material was collected and stored with the informed consent of the patients.

Control population

The sTfR was measured in 43 apparently healthy subjects (14 males and 29 females; age range=21-53 years) used as a reference population for the purpose of this study. Twelve of these subjects were randomly recruited from among the population of regular blood donors of Santo António General Hospital, Porto and 31 subjects were healthy family members of HH patients, studied as part of a routine screening program for hemochromatosis at the Predictive and Preventive Genetic Centre, Porto. Homozygosity for the C282Y allele was excluded in all control subjects. The laboratory studies for all subjects included a complete blood cell count and biochemical parameters of iron status. Informed consent for the study was obtained from all recruited subjects.

Laboratory parameters

Blood counts and iron parameters were determined by standard automatic methods routinely used at the Haematology Laboratory and Clinical Chemistry Laboratory of Santo António General Hospital, Porto. The sTfR was measured by an automatic immunoturbidimetric assay COBAS INTEGRA 700 system using a commercially available kit (Tina-quant→Soluble Transferrin Receptor, Roche). In 13 random control cases, the sTfR test was performed in duplicate in freshly collected samples and samples that had been frozen at -20°C; no significant alterations were observed in results obtained after freezing (data not shown).

Statistical analysis

Correlations between variables were investigated by simple regression analysis. For the purpose of describing variations of the several parameters as a function of transferrin saturation levels, piecewise linear regressions were run for several breakpoints in controls and patients separately. The breakpoints chosen and
shown in figures were selected by the best-fitted models within each group. Group means were compared by Student’s t-test, or ANOVA when multiple groups were compared. Distribution fitting was tested by the Kolmogorov-Smirnov method. For the purpose of analysis, logarithmic transformation was applied to the values of serum ferritin, since these have a logarithmic distribution. However, the non-transformed values are presented in Table 1. All p values are two-sided and 0.05 was taken as the level of statistical significance. Data were analyzed by StatGraphics software (Statgraphics Statistical Graphics System, version 4.0).

**Results**

**Control population (Figure 1)**

The control population consisted of 14 males and 29 females with ages ranging from 21 to 53 years. The average hemoglobin values (in g/dL) were 14.9±1.3 (range=12.3-16.6) in males and 13.1±0.8 (range=11.9-15.7) in females. TfSat values (in %) varied from 20 to 46 (mean=32±9) in males and from 10 to 48 (mean=26±10) in females. The sTfR values (in mg/L) varied from 1.84 to 5.74 with no significant differences between males and females (respectively 2.8±0.6 and 3.1±0.9). These values were normally distributed (estimated overall statistic DN=0.15; p=0.274). No significant differences were found with age. In order to analyze the relationship between the sTfR values and the other hematologic parameters, linear regression analyses were run between the sTfR and haemoglobin (Hb), mean corpuscular volume (MCV), transferrin saturation (TfSat) and the log of serum ferritin. The results show that sTfR is strongly correlated with all except the Hb values (see Table 1). As expected, the most significant correlation was found for ferritin. In addition, a similarly strong correlation was found for TfSat. This finding led us to test all other parameters in relation to the TfSat values. Figure 1 illustrates the distribution of the hematologic parameters in relation to the TfSat values. Piecewise linear regressions were run for several breakpoints of transferrin saturation. Three breakpoints (20%, 25% and 30%) were chosen a priori by inspection of the scatter plot for the whole range of TfSat, and linear regressions were run for values under or above the respective breakpoints. The best-fitted

<p>| Table 1. Correlation between the soluble transferrin receptor and other hematologic parameters in the control population. The R², r and p values are shown for each regression. |
|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Soluble Transferrin Receptor</th>
<th>Hgb</th>
<th>MCV</th>
<th>TfSat</th>
<th>Ferritin (Log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R squared (R²)</td>
<td>0.08</td>
<td>0.17</td>
<td>0.22</td>
<td>0.28</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>-0.28</td>
<td>-0.41</td>
<td>-0.47</td>
<td>-0.53</td>
</tr>
<tr>
<td>Significance level (p)</td>
<td>0.068</td>
<td>0.006</td>
<td>0.0016</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

**Figure 1.** Regression analyses between the transferrin saturation (TfSat) values and the values of hemoglobin (Hb), mean corpuscular volume (MCV), serum ferritin and soluble transferrin receptor (sTfR) in normal controls. Significant correlations are indicated by a full regression line. Dotted regression lines represent non-significant correlations.
models were obtained for the breakpoint of 25%. For TfSat values lower than 25%, significant positive correlations were found between TfSat and the values of Hb ($R^2=0.30; r=0.55; p=0.033$), MCV ($R^2=0.41; r=0.64; p=0.010$) and serum ferritin ($R^2=0.41; r=0.64; p=0.010$), whereas no significant correlations were found for TfSat values greater or equal than 25%. The sTfR, in contrast, increased significantly with decreasing TfSat values below 25% ($R^2=0.35; r=-0.59; p=0.019$), but remained at stable low levels for TfSat values from 25 to 50%. No TfSat values higher than 50% were observed in the control population.

**HH patients (Figure 2)**

In the light of the results described above in the control population, hematologic parameters were analyzed in HH patients also in relation to the TfSat values (Figure 2). For the whole range of TfSat values, highly significant positive correlations were found for MCV ($R^2=0.27; r=0.52; p=0.0003$) and serum ferritin ($R^2=0.59; r=0.77; p<0.0001$). In the case of Hb and sTfR, piecewise linear regressions were also run for several breakpoints of transferrin saturation. Three breakpoints (25%, 40% and 50%) were chosen a priori based on the model obtained in controls and by inspection of the scatter plot for the whole range of TfSat. The best-fitted model was obtained for the breakpoint of 50%. Hb values decreased significantly with decreasing values of TfSat, when these were lower than 50% ($R^2=0.35; r=0.59; p=0.006$), while a negative correlation was found for TfSat greater or equal than 50% ($R^2=0.18; r=-0.43; p=0.043$). This resulted in significantly lower Hb values in the group of patients with TfSat>70% than in the group of patients with TfSat between 50 and 70% (respectively 13.7±1.5 and 15.3±0.4; $p=0.024$). The sTfR values were also dependent of TfSat. They increased significantly with decreasing TfSat values below 50% ($R^2=0.56; r=-0.75; p=0.0002$), but remained at stable low levels for TfSat values between 50 and 100%.

Since the regression analyses illustrated in Figure 2 for the whole range of TfSat values were predicted from a group of independent subjects, it was important to confirm whether the predictions could be applied in individual cases. For this purpose, regression analyses between the TfSat and sTfR were run for serially determined values in two individual patients during the course of phlebotomy treatment. As illustrated in Figure 3, for TfSat values under 50%, highly significant correlations were confirmed in both the male ($R^2=0.90; r=-0.95; p=0.0131$) and the female ($R^2=0.87; r=-0.93; p=0.00003$) patients, fitting the regression model predicted for the whole group.

**Figure 2. Regression analyses between the transferrin saturation (TfSat) values and the values of hemoglobin (Hb), mean corpuscular volume (MCV), serum ferritin and soluble transferrin receptor (sTfR) in C282Y homozygous HH patients. Significant correlations are indicated by a full regression line. Dotted regression lines represent non-significant correlations.**
Soluble transferrin receptor in hereditary hemochromatosis

of patients and not the regression line predicted for the normal subjects (red line in Figure 3). The sTfR values were further analyzed serially for a follow-up period of six months during intensive phlebotomy treatment in one patient who presented with anemia. The results are shown in Figure 4 in comparison with the serial values of Hb and TfSat. A progressive increase in Hb values was observed in the course of the first 14 weekly phlebotomies, together with an increase in the sTfR and decrease in TfSat.

Comparisons between HH patients and controls (Table 2)

For the purpose of comparing the hematologic parameters in patients and controls, subjects were divided into groups according to the range of TfSat values (10-25%; 25-50%; ≥50%). As shown in Table 2, for TfSat values ranging from 10% to 50%, HH patients did not differ significantly from controls, although the mean value of sTfR was higher in HH patients than in controls within the group with TfS at 10-25%. For TfSat values greater or equal than 50%, HH patients had significantly higher values of MCV and serum ferritin, and significantly lower values of sTfR (see Table 2).

Discussion

In clinical practice, measurement of the sTfR is used for the diagnosis of iron deficiency as an additional parameter to other conventional laboratory tests of iron status, with the advantage that it is not influenced by acute phase responses. Furthermore, it is a non-invasive and sensitive means of evaluating bone marrow iron stores providing a good alternative to bone marrow aspirate examination, an invasive procedure that can be inadequate for interpretation in 35% of cases. Besides its clinical value in iron deficiency, the sTfR level is also an indicator of erythropoietic activity. Low values of sTfR are observed in hypoplastic erythropoiesis, and increased values are seen in erythroid hyperplasia, independently of iron stores. The finding of increased values of sTfR in secondary iron overload associated with erythroid hyperplasia led, some time ago, Cazzola and co-workers to suggest that, in these situations, the increased sTfR, through its interaction with HFE, could be one potential determinant of the secondary iron overload, suggesting a role for sTfR in iron absorption. This hypothesis has never been tested. The present study of sTfR in healthy subjects and in HH patients provided an opportunity to gain insight into the receptor’s biological significance outside the setting of iron deficiency or ineffective erythropoiesis. The results in normal subjects (Figure 1) show that the levels of circulating transferrin receptors are significantly correlated with the transferrin saturation level, reflecting the systemic effect of iron avail-

![Figure 3. Correlation between the individual soluble transferrin receptor (sTfR) and transferrin saturation (TfSat) values in two C282Y homozygous HH patients (sex and age indicated). The regression analyses were run with values obtained at different times of treatment. The regression lines obtained in each patient are represented as blue lines and compared with the regression line obtained in the group of normal controls (red lines).](image)

Table 2. Comparison of hematologic values between HH patients and controls, in groups defined according to different levels of transferrin saturation.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Controls</th>
<th>HH patients*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TfSat [10-25%] (n=15)</td>
<td>TfSat [25-50%] (n=28)</td>
</tr>
<tr>
<td>Hgb (g/dL)</td>
<td>13.2±1.0 (12.3-15.9)</td>
<td>14.0±1.4 (11.9-16.6)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>87±6 (77-97)</td>
<td>91±4 (82-98)</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>46±51 (2-187)</td>
<td>115±204 (7-880)</td>
</tr>
<tr>
<td>sTfR (mg/mL)</td>
<td>3.45±1.06 (2.26-5.74)</td>
<td>2.73±0.54 (1.84-4.08)</td>
</tr>
</tbody>
</table>

*Only one patient was excluded for this table for having TfSat <10%; *significantly different from controls (p<0.001); **significantly different from all other groups (p<0.05); ***significantly different from all other groups (p<0.004).
ability on erythropoietic activity in a normal physiological steady state. For practical purposes, a normal level of circulating sTfR should be predicted in the context of the individual TfSat level (Table 2 and Figure 1). A TfSat of 25% appeared to be the threshold value below which there was a progressive increase in sTfR, in parallel with decreases in Hb, MCV and serum ferritin in normal controls. This reflects a critical value of TfSat that could determine the up-regulation of sTfR before the development of iron-deficient anemia. For TfSat values above 25%, the sTfR is stable in a normal range, as are Hb, MCV and serum ferritin levels. This homeostatic equilibrium seems, in turn, to control the TfSat itself, since this is also stabilized at values between 25% and 50% without further increase. This control is supposed to be mediated by the regulation of iron absorption.

The idea that the erythropoietic activity, as clinically expressed by the levels of sTfR, is a determinant in the regulation of iron absorption led us to test the hypothesis that this physiological mechanism could somehow be abnormal in HH. In this context, we measured the sTfR in HH patients, all homozygous for the C282Y mutation, who had a large range of transferrin saturation values. The results show that the critical value for the up-regulation of sTfR in these patients is not 25% but may be as high as 50%. The increase in sTfR for higher TfSat values suggests that the sensing of iron available for erythropoiesis is somehow altered, behaving in iron-loaded conditions as it would in iron depletion. The consequence of this may be an increase in iron absorption that, in turn, would contribute to the increase of TfSat and perpetuation of this cycle. Probably this is not the case in other conditions of mild to moderate non-HFE hemochromatosis, in which TfSat values are not increased as in HFE-linked HH. Taking into account the fact that the appearance of marrow erythroid cells, rates of erythroid cell production and destruction, and hemoglobin are normal in HH, it can be assumed that inappropriately high levels of sTfR are not due to increased erythrocyte turnover, with or without ineffective erythropoiesis.

The observation that the threshold for the up- or down-regulation of sTfR is altered in HH is further supported by the data on the follow-up of two patients who show individual correlations fitting well with the predicted model (Figure 3). The model is compatible with the prediction that there is increased iron uptake and hemoglobin synthesis by erythroid cells in HH, as described before by Barton and co-workers. For TfSat values above 50% the sTfR is stabilized or down-regulated, thus limiting the iron uptake and further increase in hemoglobin synthesis. In these circumstances, the excessive iron available does not lead to a corresponding increase in hemoglobin (on the contrary, it decreases) but, instead, leads to iron accumulation in tissues as reflected by the increasing serum ferritin. This explains the finding of lower Hb values in patients with the highest TfSat (>70%), a situation that can be corrected by treatment, as illustrated by the follow-up of one heavily iron-overloaded patient who presented with anemia, and whose Hb increased in the course of intensive phlebotomy treatment, with a decrease in TfSat and an increase in sTfR (Figure 4).

The hypothesis of altered iron sensing by a mutant HFE protein is compatible with the specificity of certain organs or cells that are targets for iron overload in HH, and the lack of iron in other cell types such as macrophages and intestinal epithelial cells. According to the molecular model proposed by Townsend and Drakesmith, the HFE protein has two mutually exclusive activities in cells: inhibition of iron uptake (through binding to the transferrin receptor, in competition with transferrin) or inhibition of iron release. According to their model, the balance between TfSat and sTfR concentrations determines which of these functions predominates. In this way, the intestinal
crypt cells and reticuloendothelial system may interpret the body's iron requirements and regulate iron absorption and distribution, a function that fails in HH. From a systemic perspective, it is plausible that the two functions are performed simultaneously in different cell types with the common purpose of satisfying cellular iron needs. In conditions in which iron is required, there is both an increase in iron absorption and release from reticuloendothelial cells, and an increase in iron uptake by dividing cells which express transferrin receptors. The finding of increased sTfR could be a reflection of the cells' proliferative state (which is mainly influenced by the erythroid mass, but could possibly receive contributions from other cell types such as lymphocytes). They could also contribute to the regulation of iron uptake. We speculate that this could happen through competitive binding of the transferrin receptor, either on the cell surface or in the circulation, to HFE. In conclusion, the present results support the notion that a key role of HFE in iron homeostasis is the sensing or recognition of systemic iron availability. Both the transferrin saturation (reflecting systemic iron availability) and the soluble transferrin receptor (reflecting erythroid activity) are good candidates as signals in this model.

This does not exclude a role for HFE in signaling other effector molecules. Its postulated involvement also in the regulation of hepcidin activity may justify the importance of hepcidin as a modifier of expression of hemochromatosis, as elegantly reviewed by Pietrangelo in 2004. It is possible that the HFE protein, as a MHC class I protein present in a great diversity of cell types, has even wider regulatory functions, as reviewed by Cardoso and De Sousa.

GP, JCO and MB: conception and design of the study; MB, FB, JR, IG and GP: analysis and interpretation of data; MB and GP: drafting the article. All authors: critical revision of the manuscript and final approval. The authors declare that they have no potential conflicts of interest.

Manuscript received July 6, 2004. Accepted September 16, 2004.

References
23. Cardoso C, De Sousa M. HFE, the MHC and hemochromatosis: paradigm for iron extended function for MHC class I. Tissue Antigens 2003;61:263-75.